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Origin of Osteoclasts from Mononuclear Leucocytes in Regenerating Newt Limbs[1]

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The origin of the multinucleated giant s, or osteoclasts, of bone has remained doubt since 1873 when Kölliker pro- d that they were responsible for bone ption. Most authors agree that nu- r division is rarely, if ever, seen in oclasts. In his excellent review in 9, Hancox has summarized the evi- ce for the formation of osteoclasts by ion of mononucleated wandering cells stiocytes, monocytes, macrophages), the hanism proposed earlier by Haythorn 9) and others. The view that osteo- sts arise by fusion of sessile connective ue cells (osteoblasts, fibroblasts, reti- lum cells) has received some support olliker, 1873; Arey, '19; Bloom et al.,). Von Recklinghausen ('10) believed they arose by fusion of bone and ilage cells liberated from the skeletal rix. Formation of the osteoclasts as- iated with bone resorption in regen- ing salamander limbs has been at- uted to fusion of (1) undifferentiated enchymal cells (Wendelstadt, '04), lymphocytes (Fritsch, '11) (3) osteo- sts (Hellmich, '29), (4) liberated chon- ytes (Horn, '42).

It has not been possible in the past to vide definitive evidence for any of these ories because of the limitations of the thods used to study the problem. It is possible to trace cells labeled with ated thymidine by preparing autoradio- ms of tissues fixed at appropriate in- vals after incorporation of the isotope the cells (Taylor et al., '57; Leblond al., '59; Cronkite et al., '59). The regen- ing salamander limb provides a par- larly useful system in which to study oclasts by such a technique, because suspected cell precursors can be la- electively by administration of the ope at different intervals during or be- fore regeneration. After amputation of the limb, mesenchymal cells derived by dedifferentiation of the old tissues of the stump proliferate to form a new limb. The distal portion of the bone in the stump is partially resorbed and osteoclasts appear in close association with the bone remnants 10–20 days after amputation. If the animal is given tritiated thymidine during limb regeneration, and fixed on the same day as the injection, the cells synthesizing DNA at the time of injection can be detected. If animals are injected at 5, 10 or 15 days after amputation to label the mesenchymal cells in the regenerating limbs and then fixed at daily intervals after isotope administration, the fate of the labeled mesenchymal cells can be ascertained. If the animal is given tritiated thymidine before amputation of the limb, the only cells labeled are blood and epithelial cells in their sites of origin (O'Steen and Walker, '60, '61; Hay and Fischman, '61). In such animals, the labeled white blood cells that emigrate from the blood stream into the tissues of regenerating limbs can be traced by fixing the limbs at appropriate intervals after amputation. It is concluded from the results to be presented here, that the osteoclasts arise by fusion of mononuclear leucocytes, probably monocytes.

MATERIALS AND METHODS

The observations reported here were made in the course of an autoradiographic study of regenerating limbs which has previously been published in part (Hay and Fischman, '61). The techniques used and the general histology of the limbs studied were reported in detail in that publication. In brief, the forelimbs of newts (*Triturus*

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TABLE 1

Number of days before (—) or after amputation

Group number	Thyimidine-H³ administration	Fixation	Labeled cells present in the inner limb stump		
			Mesenchymal cells	Leucocytes	Osteoclasts
I	1, 2, 3, 4, 5, 8, 10, 12, 15, 18, 20, 22, 24, 26, 28	1, 2, 3, 4, 5, 8, 10, 12, 15, 18, 20, 22, 24, 26, 28	5–28	none present	none present
II A	5	5, 6, 7, 8, 9, 10	5–10	none present	none present
II B	10	10, 11, 12, 13, 14, 15	10–15	none present	none present
II C	15	15, 16, 17, 18, 19, 20	15–20	none present	none present
III	—1	1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, 28	none present	3–20	8–20

This table summarizes the experiments and results obtained. All arabic numbers refer to the days of regeneration. In group I, the animals were fixed three hours after thymidine administration. No labeled leucocytes or osteoclasts were seen in the limb stumps of this series. In group II, animals were injected at 5, 10 or 15 days of regeneration and fixed at daily intervals thereafter. No labeled osteoclasts appeared in this series. In group III, thymidine-H3 was administered one day before amputation and the regenerating limbs were fixed at daily intervals thereafter. Leucocytes were labeled in their sites of origin, but the inner tissues of the limb stump were not labeled. The labeled osteoclasts which appeared 8–20 days after amputation derived from labeled leucocytes that appeared in the limb stump 3–20 days after amputation.

viridescens) were amputated through the distal third of the radius and ulna. The animals were injected with tritiated thymidine (5 μc divided in four hourly injections) before amputation or at daily intervals after amputation. The experiments fall into three groups with respect to time of injection and the details of treatment will be presented in the results. Two regenerating limbs were fixed on each of the days selected for study and a total of 100 limbs were examined histologically.

Limbs were fixed in Bouin's fluid, embedded in paraffin, and sectioned longitudinally at 10 μ thickness. The slides containing the mounted sections were coated with Kodak NTB-3 emulsion by the melted-emulsion technique (Messier and Leblond, '57) and left in light-tight boxes for three weeks. After development, fixation, and washing of the slides, the sections were stained through the emulsion with the Cason technique (Sidman et al., '59). A few slides were stained with hematoxylin and eosin.

RESULTS

Experiment I: Regenerating limbs fixed three hours after injection of tritiated thymidine

Group I provided information as to which cells were synthesizing DNA in preparation for mitosis at the time of isotope administration. If the nuclei of osteoclasts in the regenerating limb divide by mitosis they should appear labeled in this series. Animals were injected with tritiated thymidine at 1, 2, 3, 4, 5, 8, 10, 12, 15, 18, 20, 22, 24, 26 and 28 days of regeneration and the limbs were fixed three hours later (table 1).

Osteoclasts first appear in the limb stump on about the tenth day of regeneration. They are intimately associated with the ends of the resorbing bone and may often be found lodged in bony depressions reminiscent of Howship's lacunae. Their numbers increase gradually until a peak level is reached at approximately the eighteenth day (fig. 1). Thereafter, the osteoclast population diminishes; few are seen in the limb after 22–24 days of regeneration. At this time bone resorption has ceased, and the proliferating mesenchymal cells of the blastema are beginning to redifferentiate to form the new limb.

There was no thymidine incorporation by osteoclast nuclei. In contrast, the mesenchymal cells of the limb stump show a high degree of label after isotope administration. Figure 2 illustrates an unlabeled osteoclast surrounded by labeled mesenchymal cells in a regenerate fixed on the eighteenth day after amputation.

...itotic figures are common in mesenchy-...l cells, but no mitotic figures were ever ...served in the osteoclasts. These results ...pport the conclusion (Hancox, '49a; and ...hers) that the multinucleated state is ...achieved by nuclear division in osteo-...asts.

...periment II: Regenerating limbs ...treated with thymidine at 5, 10 ... and 15 days after amputa... tion and fixed at daily in... tervals after treatment

...Animals in Series II were divided into ...ree groups (A, B, and C). Group A was ...cted on the fifth day after limb am... tation, B on the tenth day and C on the ...fteenth day. Two limbs from each group ...re fixed on the day of injection and at ...ily intervals for five days after injection ...(table 1).

...As in Series I, the mesenchymal cells ...rived from the dedifferentiating inner ...issues of the amputated stump incorpor... tritiated thymidine. Radioactive blood ...lls did not appear in the limb tissues ...thin the five day period studied. If the ...teoclasts form by fusion of mesenchymal ..., then labeled osteoclasts should be ...in the limbs containing labeled mes...ymal cells fixed at various intervals ...r the isotope administration. No ...teoclasts in Series II showed any radio...ive labeling (fig. 3). Thus, osteoclasts ...not form by fusion of mesenchymal ...s within the period studied here. It ...ems likely therefore, that the osteoclasts ...m by fusion of some other cell type ...sent in the limb, but unlabeled by the ...cedures used in Series II.

...periment III: Regenerating limbs of ...nimals injected with tritiated thy... midine one day before amputa... tion and fixed at daily in... tervals after amputation

When tritiated thymidine is injected be... amputation, only those tissues which ...mally have a population turnover are ...ted. O'Steen and Walker ('60) have ...n that in the newt, the cells predom...ly labeled by such an injection are ...ated in the epidermis, mucosal linings, ...uliferous tubules, and blood-forming ...ues. In Series III, tritiated thymidine was administered to newts to label these cell types. The limbs were amputated the day after the injection. Two regenerating limbs were then fixed at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 20, and 28 days after amputation (table 1).

Within four-five days after amputation there was an emigration of labeled polymorphonuclear and mononuclear leucocytes into the limb stumps of animals of Series III. No labeled white blood cells were seen within the tissues of the limb in Series I or II. The grain count over the nuclei of the leucocytes of the limbs in Series III showed no significant decrease throughout regeneration, and therefore, it seems unlikely that these cells divided after entering the tissues of the limb. Radioactive mononuclear leucocytes are present in the limbs until approximately the twentieth day of regeneration, but labeled polymorphonuclear leucocytes disappear after the tenth day. Thus, the life span of some of the monocytes is at least 21 days (they were labeled the day before amputation).

On the eighth day of regeneration approximately 90% of the labeled white blood cells in the stump were mononuclear leucocytes (monocytes and lymphocytes). Leucocytes with the morphological characteristics of monocytes aggregate in groups of two or three at or near the ends of the cut bones. Subsequently, labeled osteoclasts appear in close relation to the resorbing bone (10–18 days after amputation). A whole series of morphological stages can be reconstructued from monocyte to binuclear osteoclasts to multinucleated osteoclast. It seems reasonable to conclude that the osteoclasts formed from blood cell precursors, probably from these monocytes seen in the vicinity of the cut bone. The only other labeled cells present at the time are in the epidermis and there was no evidence of a transformation of epidermis into osteoclasts.

Not all of the nuclei of any given osteoclast are labeled (figs. 4 and 5). This result is consistent with the fact that not all of the mononuclear leucocytes present in the limb are labeled. Since the animals were injected with tritiated thymidine one day before amputation, leucocytes that were not preparing to divide on the day of the injection were not labeled, but had

an equal opportunity to enter the amputation site. If the isotope had been administered over a longer period prior to amputation, a larger percentage of leucocytes would have been labeled and perhaps the majority of osteoclast nuclei would then have been radioactive.

By the twentieth day after amputation very few labeled osteoclasts are seen but a number of unlabeled osteoclasts are present within the limb. These unlabeled osteoclasts probably derived from unlabeled leucocytes that entered the limb subsequent to the initial extravasation of labeled blood cells into the limb tissue. The labeled osteoclasts remaining at 20 days have pyknotic nuclei and hyperchromic cytoplasm. These morphological features are characteristic of dying cells (Hancox, '49a) and it seems likely that most of the osteoclasts which formed between the tenth and eighteenth days have degenerated or left the stump by the twentieth day of regeneration. These results suggest that the lifespan of the osteoclast in the tissues of the regenerating newt limb is normally less than ten days.

DISCUSSION

The results of the present experiments provide new evidence for the hypothesis that the multinucleated osteoclasts of vertebrates arise by the fusion of mononuclear leucocytes. The three experiments, considered together, provide compelling support for an hematogenic origin of osteoclasts in the salamander. In the first experiment, it was demonstrated that the nuclei of osteoclasts do not synthesize DNA, and no mitosis was observed in the osteoclasts. It seems reasonable to conclude, therefore, that osteoclasts do not become multinucleated as a result of division of nuclei within the cell. Tonna ('60) has studied incorporation of tritiated thymidine in rats and mice and has also reported that osteoclasts do not synthesize DNA.

In the second experiment, the undifferentiated mesenchymal cells of the limb blastema were labeled with tritiated thymidine at a time when osteoclasts were forming around bone that had begun to be resorbed. These actively growing mesenchymal cells were in close relation to the developing osteoclasts, yet the osteoclasts did not become labeled in subsequent days. Thus, the osteoclasts that formed in the limb were evidently not derived from undifferentiated mesenchymal cells of the blastema.

The third experiment provided positive evidence for the origin of osteoclasts from blood cells. Salamanders were injected with tritiated thymidine before the limb was amputated, that is to say, before any change associated with bone resorption had begun, and before any of the mesenchymal blastema cells had formed. The principal labeling observed in such animals was in blood cells and epithelium. The limbs were amputated the day after administration of isotope and fixed at daily intervals thereafter. When osteoclasts developed at the areas of bone resorption in the amputated stumps (10–20 days after amputation), *almost every osteoclast contained one or more labeled nuclei.* It seems unlikely, indeed it has never been proposed to our knowledge, that osteoclasts derive from epithelium. The only other source of labeled cells in the limb stump was the blood cell population that emigrated into the tissues after amputation.

Before and during osteoclast formation, numerous labeled mononuclear cells with the typical morphological characteristics of monocytes can be seen in the extravascular tissues of the blastema in Series III. Cells with the same morphological features appear partially fused as binuclear "osteoclasts" and a whole series of stages can be reconstructed from the mononuclear monocyte to the typical multinucleated osteoclast. Labeled polymorphonuclear leucocytes are not very numerous in the regenerate at the time osteoclasts form. Morphological considerations of the nuclear and cytoplasmic characteristics of the osteoclasts make it seem unlikely that any of the labeled osteoclast nuclei derive from polymorphonuclear leucocytes. Lymphocytes, however, are so difficult to distinguish from monocytes that a contribution from this source cannot be ruled out on morphological grounds alone.

The possibility that monocytes are the precursors of osteoclasts in mammals as well as amphibians is consistent with the

known phagocytic properties of mono- and their ability to transform readily macrophages (Carrel and Ebeling,). There is abundant evidence in the erature to suggest a physiological rela- ship between monocytes, macrophages osteoclasts. The undulating mem- e (Hancox, '49b), active motility (Han- '46), and affinity for supravital neu- red (Barnicot, '47), are characteristic perties of macrophages shared by osteo- s. Osteoclasts differ from classical rophages in that they fail to take up an blue in vital staining studies (Ship- and Macklin, '16). Phagocytosis of red d cells has, however, been attributed steoclasts (Arey, '19; and others). Re- ly, histochemical studies have shown acid phosphatase activity in macro- es. Indeed, the entire reticuloendo- al system can be demonstrated by this chemical technique and it seems that all phagocytes contain this some enzyme (Barka et al., '61). acid phosphatase activity has been ted in osteoclasts (Burstone, '60). It teresting to note here that osteocytes osteoblasts have low acid phospha- but high alkaline phosphatase con- rations. Foreign body giant cells, inuclear phagocytes that probably e by fusion of monocytes (Haythorn,), also have high acid phosphatase ac- y (Burstone, '60). Electron micro- e investigations by Scott and Pease) and Gonzales and Karnovsky ('61) est an active "phagocytosis" of bone by the osteoclast. In view of the iological properties shared by macro- es and osteoclasts, it is not surprising the present autoradiographic evi- e suggests that osteoclasts arise by n of monocytes in amphibians, and tempting to think that a similar mech- m occurs in other vertebrates.

The present study demonstrated that nonuclear leucocytes in the newt can at least 21 days (from the time of in- ration of the isotope until departure the leucocytes from the limb). Com- ble figures are not available for mam- n mononuclear leucocytes. Hancox) has suggested that the lifespan of osteoclast *in vitro* is less that 48 hours. finite turnover of osteoclasts *in vivo* was demonstrated in the present study. Labeled osteoclasts in the amphibian regenerate were replaced within about ten days after they had formed. A further point of some interest in the present investigation is the fact that no labeled white blood cells were seen within the limb tissues in Series I and II. The results of Series I indicate that leucocytes do not synthesize DNA after entering the limb tissues. The results of Series II indicate that developing leucocytes labeled by injection at 5, 10 or 15 days following amputation did not enter the limb within the five day periods after injection which were analyzed. These results are compatible with the conclusion that the maturation period of newt leucocytes (expressed in terms of time between incorporation of thymidine and ability to enter the limb tissues) is normally greater than five days.

SUMMARY

The origin of the osteoclasts in regenerating forelimbs of *Triturus viridescens* was studied by autoradiography using tritiated thymidine. When animals were injected with the labeled nucleoside and their regenerating limbs fixed the same day, no radioactive osteoclasts were detected. Thus, it was concluded that osteoclasts do not divide by mitosis. When animals were injected at 5, 10 or 15 days of regeneration and their limbs fixed at daily intervals for five day periods, no labeled osteoclasts were seen. The labeled mesenchymal cells of the blastema did not fuse to form osteoclasts in the five day periods studied. When animals were injected one day before amputation and limbs fixed at various stages of regeneration, labeled osteoclasts were seen in the limb stumps between the tenth and twentieth days after amputation. Prior to the appearance of the labeled osteoclasts, the only radioactive cells in the inner limb tissues were extravascular blood leucocytes. It is concluded that osteoclasts of the regenerating salamander limb form by fusion of mononucleated leucocytes, probably monocytes.

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PLATE 1

EXPLANATION OF FIGURES

All photomicrographs are of autoradiographs of sections of regenerating forelimbs of *Triturus*. The exposed silver grains visible over radioactive nuclei are in the emulsion over the section.

B, Bone
mes, mesenchymal cell
Os, Osteoclast

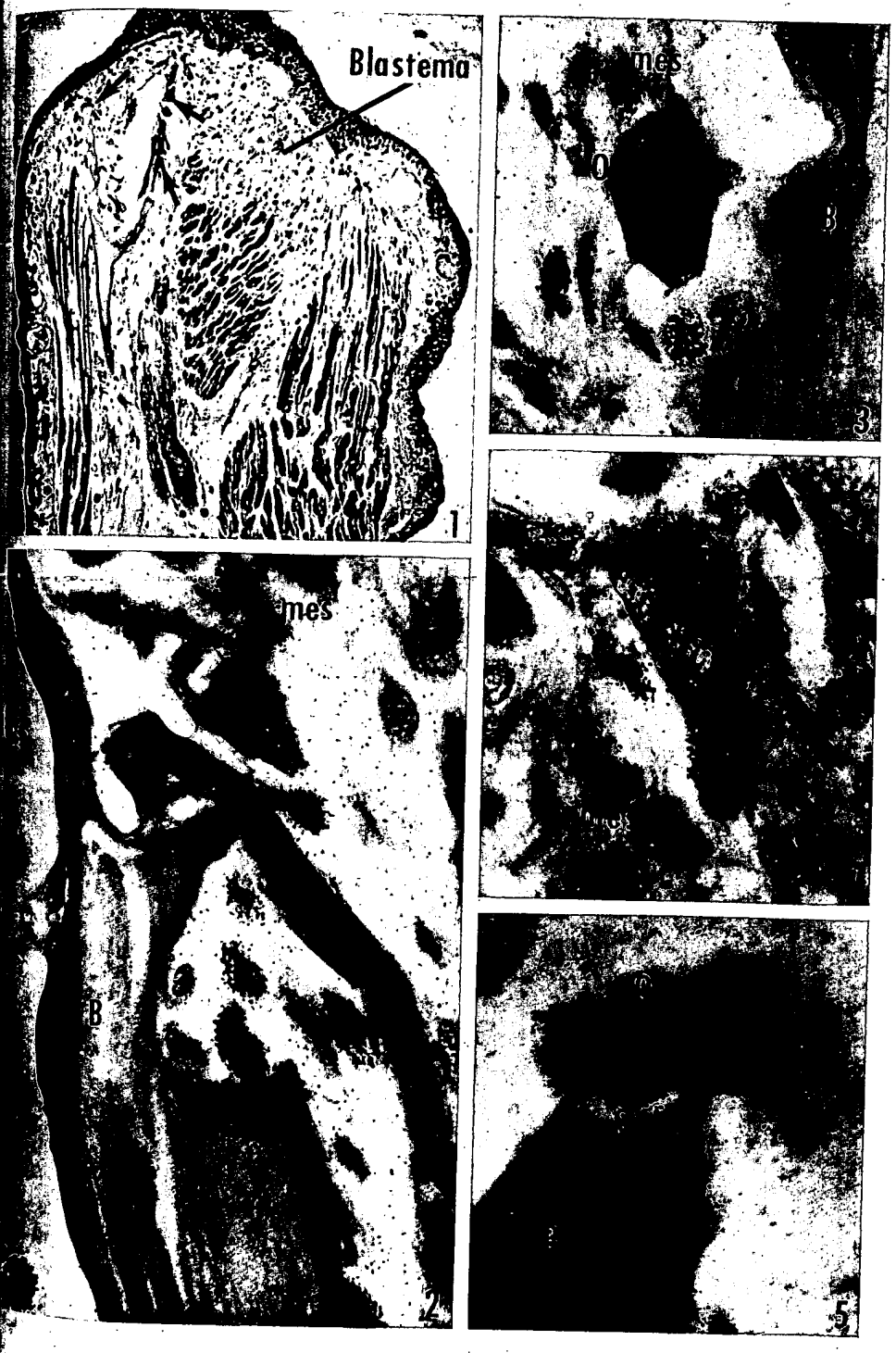
1 Low power photomicrograph of a section from an 18 day regenerate in Series I. Arrows point to osteoclasts located at the distal end of transected bone. Mesenchymal cells have begun to accumulate at the distal end of the stump to form the blastema.

2 Higher magnification photomicrograph of a section from an 18 day regenerate in Series I. The animal was injected with tritiated thymidine on the eighteenth day of regeneration and the limb stump fixed three hours later. This experiment was designed to label those cells synthesizing DNA during regeneration. Note the unlabeled osteoclast surrounded by labeled mesenchymal cells. Because of absence of DNA synthesis in osteoclasts, it was concluded that osteoclast nuclei do not divide. 450 ×.

3 This section is from a 14 day regenerate in Series II. The animal was injected with tritiated thymidine on the tenth day of regeneration and the limb fixed four days later. This experiment was designed to follow labeled mesenchymal cells in the blastema of regenerating limbs. Note the unlabeled osteoclast surrounded by labeled mesenchymal cells. Because labeled osteoclasts did not appear in Series II, it was concluded that osteoclasts do not form by fusion of the mesenchymal cells of the blastema. 450 ×.

4 This section is from a 15 day regenerate in Series III. The animal was injected with tritiated thymidine one day before amputation to label leucocytes in their sites of origin. No labeling occurs in the inner tissues of the unamputated limb. Labeled leucocytes appear in the regenerate shortly after amputation and soon thereafter labled osteoclasts can be found. The osteoclast shown in this field contains two radioactive nuclei. The unlabeled nuclei probably arose from leucocytes not labeled at time of injection. The clump of grains to the left of the osteoclast lies over the nucleus of a leucocyte which is not shown clearly in the section. From the results in Series III, it was concluded that osteoclasts form by fusion of leucocytes. 550 ×.

5 This is another section from a 15 day regenerate in Series III treated as explained in the legend for figure 4. The labeled nuclei of the osteoclast (Os) were derived from leucocytes labeled before amputation of the limb. 550 ×.


Blastema
1
mes
B
3
mes
B
2
5

PLATE